Title: **CHRONIC CLAM (MERCENARIA MERCENARIA) TOXICITY TEST**

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1.0 OBJECTIVE

This method measures the chronic toxicity of chemicals or whole sediment to the marine bivalve, *Mercenaria mercenaria*, during a ten-day, static exposure. The endpoint measured is mortality. This protocol is adapted from Chung 1999, Fulton et. Al, 1999, and Ringwood et al., 1996.

2.0 HEALTH AND SAFETY

Personnel should wear lab coats, lab aprons, safety goggles, and chemical resistant gloves when preparing chemical stocks, and when dosing with test chemicals or effluents.

3.0 PERSONNEL/TRAINING/RESPONSIBILITIES

This method should be restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

4.0 REQUIRED AND RECOMMENDED MATERIALS

Revco® Environmental chamber
Analytical balance
Stainless steel 212-µm sieves
600-ml glass beakers
50-ml beaker
Volumetric pipets
Pipet bulbs
Stainless Steel Spoons
1 Liter Volumetric flasks
16-oz glass jars
Aluminum Foil
YSI 55
pH meter
Refractometer

pH meter	Disposable Petri dishes
Dissecting Microscope	Label Tape
Aquarium pumps	Aquarium Tubing
Gang Valves	Nitrile Gloves
Falcon® 3-ml plastic transfer pipets	Falcon® 60 x 15-mm polystyrene petri dishes
Sodium Dodecyl Sulfate	1-L Graduated Cylinders
Aluminum Weighing Dish	Parafilm®

5.0 PROCEDURE

5.1 Preparation

5.1.1 Sediment Collection

Collect sediment from test sites using the methods listed in SOP 00-???. Sediment used for spiking will be collected from a relatively uncontaminated site on the Folly River. The coordinates for the control site is **32° 15.23 N and 80° 52.23 W**. Collect the sediment at least three days prior to the start of the test. Sediment should not be used if it is past 30 days from collection date.

Upon arrival back at the laboratory, homogenize the sediment and aliquot out 8 oz for Archive in the chemistry database. Aliquot out the rest of the sediment for the clam assay (fill 1-gallon jar with sediment).

Any unused sediment can be disposed of in the trash (not in the sink).

Once all the sediment has been collected, prepare to sieve the sediment (4-5 days prior to the start of the test).

Rinse the 212-µm sieves with acetone prior to sieving. Use sieves labeled "CONTROL" for Folly sediment.

When sieving, be certain to wear an apron, gloves, and protective eyewear. Place the sieve on top of a stainless steel pot. Using a pre-cleaned spoon, homogenize the sediment in the jar and spoon out into the sieve. Pull out any visible detritus or debris (grass blades, pine needles, shells, snails, broken glass, etc…) and using a 250-ml Volumetric flask with an etched bottom or your hand, gently pushed the sediment through the sieve. Try not to add 20-µm filtered seawater (in a squirt bottle) unless the sediment is extremely dry and not going through the sieve. Make sure to rinse the sieves out between sites with acetone.

Using a solvent rinsed stainless steel spoon, transfer the sieved sediment into a pre-cleaned gallon jar. Label the jar with the word "sieved" on the lid.

Once all the sediment has been sieved, return the jar to the walk-in freezer.

5.1.1 Water collection and Storage

The water used for the acute clam assay is collected from Bohicket Creek, a relatively uncontaminated tidal tributary of the North Edisto River Estuary. See the water collection SOP for specifics.

Allow the collected natural seawater to settle for 24-48hrs. Check the salinity of the seawater. It must be 30-ppt or higher salinity to conduct the experiment.

After the particles in the water have settled, filter the water through a 20-µm filter.

If testing is to be conducted in Room 404, then the filter seawater needs to be transferred from the holding containers in Building 500.

5.1.2 Collection and Holding of *Mercenaria mercenaria*

Test organism - *Mercenaria mercenaria*, an estuarine bivalve.

Juvenile clams are acquired from Sea Perfect Inc. located on Folly Road (James Island). This is approximately 15-20 minutes away from the laboratory. Clams are placed in a 16-oz jar with water from the clam farm.

Upon arrival back at the laboratory, check the salinity of the seawater from the jar. The reading should be around 28-30ppt. Bring the salinity of the jar up to 30ppt by adding filtered seawater from the carboy in Room 404.

Clams are sieved through a 212-µm sieve. Those retained on the sieve should be <350 and >212 µm in size. This can be done at the clam farm or back in the laboratory.

The juvenile clams are then acclimated for 24-48hr in a Revco Environmental Chamber in 16-oz pre-cleaned jars at 20°C, 30ppt, and a 12-h light:12-h dark cycle.

Clams are fed daily with *Isochrysis galbana* (average count of 6-8 million cells/ml) obtained from the clam farm.

Prepare for the reference test.

5.2 Reference Test

5.2.1 Reference toxicant

Sodium dodecyl sulfate (SDS) is used as the reference toxicant. Reference toxicant tests were conducted to assure that each lot of clams used in the different assays was healthy.

5.2.2 Making the stock

Range finder assays have been conducted using concentrations of 1.94, 3.24, 5.4, 9.0, 15.0 and 25.0 µg/L.

In an aluminum weight boat, weigh out 0.5g of SDS (found in Rm. 230).

Pour the powder into a clean 50-mL beaker and add deionized (DI) water to the 30-mL mark.

Place a magnetic stir bar in to the beaker and stir for a minimum of 15 minutes on the stir plate. Cover the beaker with Parafilm®.

Pour the SDS mixture into a 50-mL volumetric flask.

Rinse out the beaker and stir bar with DI water and pour the rest of the mixture in the flask.

Rinse off the sides of the flask with DI water and bring the volume up to the 50-mL mark.

Label and date the flask.

5.2.3 Setting up the reference test

Add 300 ml of 20µm-filtered seawater (30ppt) to each labeled beaker.

Add appropriate amount of SDS and DI water to each beaker for each concentration (Table 1).

Bring volume up to 500 ml.

Add 10 clams to each beaker and cover with solvent-rinsed aluminum foil and aerate with a 1-ml pipet.

Take parameters from the control beaker and place in to the Environmental chamber for 24 hours.

5.2.3 Breaking down the reference test

Take a plastic pipet and gently swirl the water in the Control beaker. The clams should pool in the center of the beaker. Clams are pipetted from their respective beakers using a Falcon® 3-mL plastic disposable transfer pipette, into labeled Falcon® 60 x 15-mm disposable polystyrene petri dishes.

Using a dissecting microscope, determine the mortality of the clams. Clam mortality is determined by visible inspection using a dissecting microscope. Clams are determined to be alive if locomotion is exhibited following placement in the petri dish or by tapping the petri dish to move clams around that remained closed for several minutes.

If the 24-h SDS LC_{50} value for a new lot of clams falls within two standard deviations of the averaged LC_{50} value, then the population of clams used for the aqueous bioassay will be considered healthy and testing proceeds.

5.3 Testing with Spiked Sediment

5.3.1 Test Concentrations

Range finding tests using a series of test chemical concentrations should be tested initially.

After a range finding test, a definitive, narrow range of test chemical concentrations are performed to delineate the dose-response curve.

5.3.2 Start of the Test

5.3.2.1 Tests using 600-mL Beakers

Prepare beakers (five replicates for each of the concentration and the control) by

following the Glassware SOP 00-???.

Using the Dremmel tool, cut enough 1-ml pipets (between the "4" and "5" ml marks) for the test plus a few extras. Make sure you have on protective eyewear.

Label 600-mL Pyrex glass beakers with the appropriate concentration, replicate and name of contaminant.

If the SDS test passes then the sediment test will proceed.

Add appropriate amount of testing compound and carrier solvents to each beaker for each concentration and control. Remember to keep the concentration of carrier solvents constant in the treatments and controls. Acetone should be kept at 0.1%. It may be desirable to confirm the chemical concentration of the stock analytically. Preparation of chemical stocks and test concentrations should be performed in a fume hood.

Add 100 ml of spiked sediment into each beaker.

Add 300 ml of filtered seawater and cover with solvent-rinsed aluminum foil and aerate with a 1-ml pipet. Allow beakers to aerate overnight before the addition of the clams.

After the sediment has aerated overnight, add 50 clams to each beaker.

Random numbers have been generated for the placement of the 600-ml beakers in the Environmental Chamber.

Take parameters from the control beaker and place the beakers in the Revco® Environmental chamber.

The bioassays are conducted at 30-ppt salinity (20-µm filtered seawater), 20°C, and a 12-h light: 12-h dark cycle.

Clams are fed 5-ml of *Isochrysis galbana* (from the clam farm) every 48 hours during the chronic (10-day) test periods.

Water quality parameters (temperature, dissolved oxygen, salinity, and pH) are recorded daily during the exposure period from each control replicate. Take a 1ml water sample from the parameter beakers for an Ammonia Test (SOP 00-???) on Days 0, 2, and 8 of the test period.

The test begins when the clams are added to the beakers.

5.3.2.2 Tests using 16-oz glass jars

Prepare jars (five replicates for each of the concentration and the control) by following the Glassware SOP 00-???.

Using the Dremmel tool, cut enough 1-ml pipets (between the "4" and "5" ml marks) for the test plus a few extras. Make sure you have on protective eyewear.

Label 16-oz glass jars with the appropriate concentration, replicate and name of contaminant.

If the SDS test passes then the aqueous test will be proceeded.

Add appropriate amount of testing compound and carrier solvents to each beaker for each concentration and control. Remember to keep the concentration of carrier solvents constant in the treatments and controls. Acetone should be kept at 0.1%. It may be desirable to confirm the chemical concentration of the stock analytically. Preparation of chemical stocks and test concentrations should be performed in a fume hood.

Add 60 ml of spiked sediment into each jar by the following method. Take a 100-ml beaker and record its weight on the scale. Tare this weight and add enough of the homogenized sediment to the 60-ml mark. Record this weight. Take the 16-oz jar and place it on the scale and tare the weight. Add the recorded weight for the "60-ml" into the 16-oz jar for that concentration. Repeat this process for each concentration.

Add 180 ml of 20µm-filtered seawater (30ppt) to each labeled beaker and cover with the lid (through with a 1-ml pipet is inserted for aeration). Allow the jars to aerate overnight before the addition of the clams.

After the sediment has settled overnight under aeration, add 30 clams to each jar.

Take parameters from the control jar and place all jars in the Environmental chamber for 24 hours.

Random numbers have been generated for the placement of the16-oz jars in the Environmental Chamber.

The bioassay is conducted at 30-ppt salinity (20-µm filtered seawater), 20°C, and a 12-h light: 12-h dark cycle.

Clams are fed 5-ml of *Isochrysis galbana* (from the clam farm) every 48 hours during the chronic (10-day) test periods.

Water quality parameters (temperature, dissolved oxygen, salinity, and pH) are recorded daily during the exposure period from each control replicate. Take 1ml water sample from the parameter beakers for an Ammonia Test (SOP 00-???) on Days 0, 2, and 8 of the test period.

The test begins when the clams are added to the jars.

5.3.3 End of the Test

The test is terminated after 10-days after initiation.

Clam mortality for the sediment test is determined in the same manner as the reference test.

The data are then analyzed using appropriate statistics and an LC_{50} is calculated.

Control jars are emptied into the trash. Treatment jars must be emptied into appropriate spent chemical containers and immediately solvent rinsed. See Glassware SOP 00-???.

5.4 Testing with Field Collected Sediment

5.4.1. Start of the Test

Collect sediment from test sites using the methods listed in SOP 00-???. Control sediment will be collected from a relatively uncontaminated site on the Folly River. The coordinates for the control site is 32°15.23 N and 80°52.23 W. Collect the control site sediment at least three days prior to the start of the test. Sediment should not be used if it is past 30 days from collection date.

Upon arrival back at the laboratory, homogenize the sediment and aliquot out 8 oz for Archive in the chemistry database. Aliquot out the rest of the sediment for the clam assay (fill 1-gallonjar with sediment).

Any unused sediment can be disposed of in the trash (not in the sink).

Once all the sediment has been collected, prepare to sieve the sediment (4-5 days prior to the start of the test).

Rinse the 212-µm sieves with acetone prior to sieving. Use sieves labeled "CONTROL" for Folly sediment and "CONTAMINANTED" sieves for all other sites.

When sieving, be certain to wear an apron, gloves, and protective eyewear. Place the sieve on top of a stainless steel pot. Using a pre-cleaned spoon, homogenize the sediment in the jar and spoon out into the sieve. Pull out any visible detritus or debris (grass blades, pine needles, shells, snails, broken glass, etc…) and using a 250-ml volumetric flask with an etched bottom or your hand, gently pushed the sediment through the sieve. Try not to add 20-µm filtered seawater (in a squirt bottle) unless the sediment is extremely dry and not going through the sieve. Make sure to rinse the sieves out between sites with acetone. Also remember to change gloves between sites.

For a five replicate test, the 32-oz jar should be half full. Label the jar with the word "sieved" on the lid.

Once all the sediment has been sieved, return the jar to the walk-in freezer.

5.4.1.1 Tests using 600-mL Beakers

Prepare beakers (five replicates for each of the concentration and the control) by following the Glassware SOP 00-???.

Using the Dremmel tool, cut enough 1-ml pipets (between the "4" and "5" ml marks) for the test plus a few extras. Make sure you have on protective eyewear.

Label 600-mL Pyrex glass beakers with the appropriate site and replicate.

If the SDS test passes then the sediment test will proceed.

Take the sediment jars out of the walk-in freezer and allow them to acclimate to room temperature before aliquotting into the beakers.

Once the sediment has reached room temperature, homogenize the sediment and add 100 ml to the beakers.

Add 300 ml of filtered seawater and cover with solvent-rinsed aluminum foil and aerate with a 1-ml pipet. Allow beakers to aerate overnight before the addition of the clams.

After the sediment has aerated overnight, add 50 clams to each beaker.

Random numbers have been generated for the placement of the 600-ml beakers in the Environmental Chamber.

The bioassays are conducted at 30-ppt salinity (20-µm filtered seawater), 20°C, and a 12-h light: 12-h dark cycle.

Clams are fed 5-ml of *Isochrysis galbana* (from the clam farm) every 48 hours during the chronic (10-day) test periods.

Water quality parameters (temperature, dissolved oxygen, salinity, and pH) are recorded daily during the exposure period from randomly selected beakers (2 from each site). Take a 1 ml water sample from the parameter beakers for an Ammonia Test (SOP 00-???) on Days 0, 2, and 8 of the test period.

The test begins when the clams are added to the beakers

5.3.2.2 Tests using 16-oz glass jars

Prepare jars (five replicates for each of the concentration and the control) by following the Glassware SOP 00-???.

Using the Dremmel tool, cut enough 1-ml pipets (between the "4" and "5" ml marks) for the test plus a few extras. Make sure you have on protective eyewear.

Label 16-oz glass jars with the appropriate site and replicate.

If the SDS test passes then the sediment proceeds.

Add 60 ml of spiked sediment into each jar by the following method. Take a 100-ml beaker and record its weight on the scale. Tare this weight and add enough of the homogenized sediment to the 60-ml mark. Record this weight. Take the 16-oz jar and place it on the scale and tare the weight. Add the recorded weight for the "60-ml" into the 16-oz jar for that concentration. Repeat this process for each site.

Using a 1-liter graduated cylinder, add 180 ml of 20µm-filtered seawater (30ppt) to each labeled jar. Insert a cut 1-ml pipet through the lid of the jar for aeration. Allow beakers to aerate overnight before the addition of the clams.

After the sediment has aerated overnight, add 30 clams to each jar.

Random numbers have been generated for the placement of the 16-oz jars in the Environmental Chamber.

The bioassays are conducted at 30-ppt salinity (20-µm filtered seawater), 20°C, and a 12-h light: 12-h dark cycle.

Clams are fed 5-ml of *Isochrysis galbana* (from the clam farm) every 48 hours during the chronic (10-day) test periods.

Water quality parameters (temperature, dissolved oxygen, salinity, and pH) are recorded daily during the exposure period from randomly selected jars (2 from each site). Take a 1 ml water sample from the parameter beakers for an Ammonia Test (SOP 00-???) on Days 0, 2, and 8 of the test period.

The test begins when the clams are added to the jars.

5.3.3 End of the Test

The test is terminated after 10-days after initiation.

Clam mortality for the sediment test is determined in the same manner as the reference test.

The data are then analyzed using appropriate statistics and an LC_{50} is calculated.

Control jars are emptied into the trash. Treatment jars must be emptied into appropriate spent chemical containers and immediately solvent rinsed. See Glassware SOP 00-???.

6.0 QUALITY ASSURANCE/QUALITY CONTROL

Personnel should follow good laboratory practices during sediment testing. The number of replicates tested should be five.

7.0 REFERENCES

Chung, K.W. 1999. Toxicity of cadmium, DDT, and fluoranthene to juvenile *Mercenaria mercenaria* in aqueous and sediment bioassays. Master's Thesis. University of Charleston. 123 pp.

Fulton, M.H., G.I. Scott, P.B. Key, G.T. Chandler, R.F. Van Dolah, P.P. Maier, and M.A. Lewis. 1999. Comparative toxicity testing of selected benthic and epibenthic organisms for the development of sediment quality test protocols. Final Report. USEPA/600/R-99/011. 47 pp.

Ringwood, A.H., A.F. Holland, R. Kneib, P. Ross. 1996. EMAP/NS&T pilot studies in the Carolinian Province: Indicator testing and evaluation in southeastern estuaries. Final Report. NOAA Technical Memorandum NOS ORCA 102. National Oceanic Atmospheric Administration, Silver Springs, MD, USA

8.0 TABLES

Table 1. Sodium Dodecyl Sulfate (SDS) test concentrations.

Concentration	**Amount of Sodium Dodecyl Sulfate added (ml)**	**Amount of DI H_2O Added (ml)**
Control	0.00	1.25
1.94	0.10	1.15
3.24	0.16	1.09
5.4	0.27	0.98
9.0	0.45	0.80
15.0	0.75	0.50
25.0	1.25	0.00